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**SALT-INDUCED AGGREGATION OF LYSOZYME: IMPLICATIONS  
FOR CRYSTAL GROWTH**

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## Introduction

Crystallization of proteins is a prerequisite for structural analysis by x-ray crystallography. While improvements in protein crystals have been obtained in microgravity onboard the U.S. Space Shuttle [1], attempts to improve the crystal growth process both on the ground and in space have been limited by our lack of understanding of the mechanism(s) involved. Almost all proteins are crystallized with the aid of a precipitating agent. Many of the common precipitating agents are inorganic salts. An understanding of the role of salts on the aggregation of protein monomers is key to the elucidation of the mechanism(s) involved in protein crystallization. In order for crystallization to occur individual molecules must self-associate into aggregates. Detection and characterization of aggregates in supersaturated protein solutions is the first step in understanding salt-induced crystallization. Laser light scattering [2], and more recently neutron scattering [3], have provided evidence for the presence of aggregates supersaturated protein solutions. However, each of these methods is severely limited in their ability to make direct measurements of the concentration of discrete aggregate species present in a polydisperse solution. Light scattering experiments, costly in both equipment and time, are further impaired by their reliance upon clean solutions for accurate measurements. This is an impediment for protein crystal growth experiments, often requiring supersaturated solutions, by their very nature unstable and subject to particulate (i.e. crystalline) contaminations.

We have used a dialysis kinetic technique to study the role of sodium chloride on the aggregation process of hen egg white lysozyme. This dynamic dialysis technique is based upon the fact that the flux of protein molecules across a porous barrier is proportional to the size, diffusivity and the concentration difference across that barrier. By analyzing the flux at which protein molecules cross a semipermeable membrane the concentrations of discrete aggregates can be calculated. Using this method we have determined the aggregate distribution of lysozyme in supersaturated solutions of varying salt content. A model incorporating the unusual protein chemistry of lysozyme has been developed to model the aggregate distribution. The aggregate distributions of the solutions have been compared to face growth rate measurements allowing identification of the growth unit.

## Materials and Methods

Materials . Hen egg white lysozyme was purchased from Calbiochem. All buffer components, except sodium phosphate monobasic (Fisher-Scientific Company), sodium chloride and 70% glutaraldehyde were all reagent grade from Sigma Chemical Company. Cellulose ester dialysis tubing was from Spectrum. Electrophoretic reagents, standards, and precast gels were from Pharmacia. Lysozyme was dissolved and dialyzed against distilled water

overnight to remove salts and buffers. It was then re-dialyzed against 1%(w/v) NaCl (0.1M NaAc, pH 4.0). All working solutions were stored in a water bath at 20°C until used. To prepare protein in 3%(w/v) NaCl (0.1M NaAc, pH 4.0), equal volumes of the working solution and 5%(w/v) NaCl (0.1M NaAc, pH 4.0). Lysozyme concentrations were determined using an A(1%, 281.5nm.)= 26.4 on a Varian DMS 200 UV-VIS spectrophotometer.

Cross-linking . The preparation of covalently cross-linked lysozyme was prepared by the addition of 200 mL of 70% glutaraldehyde to 5 mL of 20 mg/mL lysozyme in 3%(w/v) NaCl (0.1M NaAc, pH 4.0) was allowed to react for five minutes before it was loaded onto a 115 x 5 cm size exclusion column of G-50 Sephadex running at 0.2 mL/min. Fractions were collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on Pharmacia's PhastSystem using the recommended protocols. The samples were dialyzed against distilled water and lyophilized for storage.

Dialysis Kinetics . The dialysis kinetics apparatus has been described previously [4 and 5] so only pertinent items will be discussed. Briefly, the polydisperse protein solution was placed inside of a custom made dialysis bag with a membrane with a molecular weight cut-off (MWCO). Aggregates with molecular weights larger than the MWCO cannot escape the bag. In order to overcome the osmotic back flow which results in buffer flowing inside of the bag during the first 10 minutes of a run pressure was kept on the bag using a syringe. The dialysis bag was placed inside of a thermostated beaker within which water was circulated, via a circulating bath, in order to maintain the temperature at 20°C. Approximately 115 mL of buffer, also kept at 20°C, was placed in the beaker for each run. The external buffer solution was circulated through an ISCO Model 229 UV-VIS detector monitoring the absorbance at 281.5 nm. The output voltage of the detector was recorded using an NB-MIO-16X multi-purpose acquisition board in a Macintosh II cx computer running LabView.

## Results

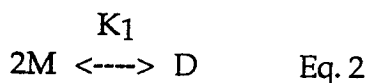
Development of a dimerization model. In order for this technique to be successful we must be certain of which aggregates are retained in the dialysis bag and which can escape through the pores. Our approach was to prepare standard solutions of aggregates of hen egg white lysozyme. The aggregates were cross-linked with glutaraldehyde and then separated on a G-50 size exclusion chromatography column. The purity of the aggregates was checked with SDS-PAGE which separates proteins by their molecular weights. The flux of purified dimer from a 25K MWCO dialysis membrane was found to be 0.15 sec<sup>-1</sup> in 1% NaCl (pH 4.0, 0.1M NaAc). This is about 30% of the monomer flux rate of 0.50 sec<sup>-1</sup> in the same buffer.

Knowing this we constructed a model which describes the total flux,  $F_{tot}$ , from the membrane to be composed of monomer concentration,  $[M]$ , times the

flux of the monomer,  $F_{\text{mono}}$  plus the dimer concentration,  $[D]$  times the flux of the dimer,  $F_{\text{dimer}}$ .

$$F_{\text{tot}} = [M]F_{\text{mono}} + [D]F_{\text{dimer}} \quad \text{Eq. 1.}$$

Since  $F_{\text{mono}}$  and  $F_{\text{dimer}}$  are known to be  $0.50 \text{ sec}^{-1}$  and  $0.15 \text{ sec}^{-1}$  respectively and  $F_{\text{tot}}$  can be measured we can get monomer and dimer concentrations from the following equilibrium:



where  $K_1$  is equal to:

$$K_1 = \frac{[D]}{[M]^2} \quad \text{Eq. 3.}$$

Assuming that the total concentration is composed of monomers and dimers we can obtain  $K_1$  estimates by measuring the total flux,  $F_{\text{tot}}$ , and using non-linear least squares regression analysis (MINSQ) to vary the monomer and dimer concentrations until an optimum fit is obtained.

Flux rate measurements and estimation of dimerization constant. The total flux from a 25K MWCO dialysis membrane was measured at four different salt concentrations. These flux measurements were found to vary significantly with the supersaturation level of the solution (Fig. 1).

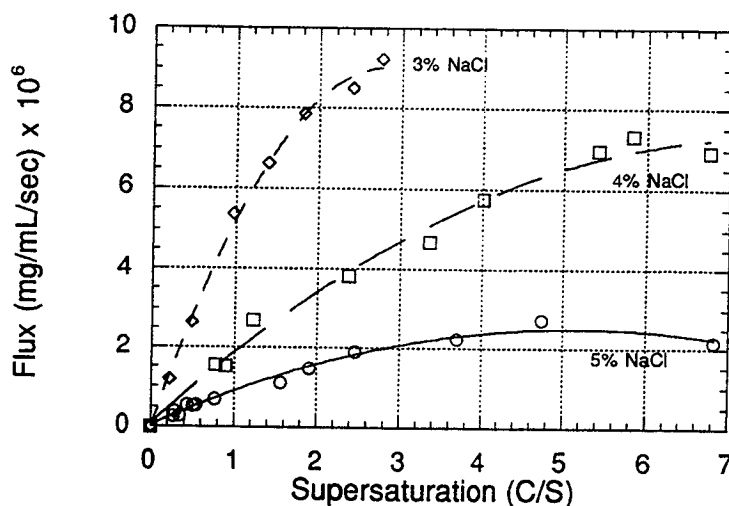


Figure 1. Flux measurements as a function of supersaturation.

Using the dimerization model the value of  $K_1$  was estimated (Table 1) and the goodness of fit was tested by a graph of the calculated flux vs. the observed

flux. As expected the model fit the data well at low protein concentrations. However, at high concentrations the observed flux showed large curvature indicating the presence of higher ordered species. Since our initial model only allows for monomers and dimers we would not expect good agreement when higher aggregates are present.

Table 1. Equilibrium Constants for 1->2 Pathway

% NaCl	Solubility (mg/mL) [6]	K <sub>1</sub>	R <sup>2</sup>
1	>100	2.4844	0.99728
3	7.14	98.09	0.98719
4	2.43	290.39	0.98840
5	1.69	526.15	0.98012

There was a dramatic increase in K<sub>1</sub> at salt concentrations greater than 3% NaCl. Since the solubility of lysozyme decreases with increasing salt concentration the supersaturation levels are higher for higher salt concentrations. However, the increase in K<sub>1</sub> was not linear with the solubility or with the salt concentration. Instead it increased exponentially with salt concentration above 3% NaCl. The most obvious conclusion from the data in Table 1. is that the salt is driving the aggregation process.

## Conclusions

The simple model we have used assumes that the first step in the aggregation process is the formation of a dimer from two monomers. Using this model we have determined equilibrium constants for this step which allows us to calculate monomer and dimer concentrations. When we compare how the dimer concentrations vary over a given supersaturation range we see an increase in the amount of dimer. In fact the profile of the dimer concentration parallels the profile of measured 110 face growth rates [7]. In contrast, the monomer concentrations level out at supersaturations where the crystal is going at a significant rate. This leads one to assume that aggregates, and not monomers, are responsible for the growth of the crystals. Also, the high level of aggregated species in solutions which lead to crystal growth must affect both the nucleation and growth mechanisms.

It is recommended that future efforts be directed toward the development of a model which incorporates more aggregation steps. Of particular interest is the next step which could be a) the formation of trimers from a dimer and a monomer or b) the formation of tetramers from two dimers. Both of these scenarios have been seen in aggregating biological systems. In order to determine this critical next step it will be necessary to use a 50K MWCO membrane which allows trimers, if present, to escape.

Additional improvements in the purification of the cross-linked aggregates is necessary to allow characterization of dialysis membranes more easily and accurately. Knowing the escapable units for each MWCO is a requirement for development of appropriate models and determination of equilibrium constants for each step in the pathway up to a critical nucleus.

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